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ABSTRACT

A method for treating a solid tumour or cancer in a subject, the method comprising administering to the subject an effective amount of a Coxsackie A group virus or a modified form thereof such that at least some cells in the tumour are killed by the virus.

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COMPLETE SPECIFICATION

Invention Title:

Method of Treating a Malignancy in a Subject

The invention is described in the following statement:

Field Of The Invention

The present invention relates to methods of treating solid tumours or malignancies, and pharmaceutical compositions for use in same. The invention finds veterinary use as well as broad application in the human medical field.

5

Background Of The Invention

Viruses capable of inducing lysis of malignant cells through their replication process are known as oncolytic viruses and trials using oncolytic viruses to treat malignancies have been performed (Nemunaitis J; 1999). Most oncolytic viruses require 10 proliferation in the same species or cell lineage. Infection of a cell by a virus involves attachment and uptake into the cell which leads to or is coincidental with uncoating of the viral capsid, and subsequently replication within the cell (Fenner F., et al. The Biology of Animal Viruses. Academic Press. New York, 1974 Second Ed.)

Oncolytic viruses assessed for capacity to kill cancer cells have included the 15 adenovirus subtype Egypt 101 virus which showed oncolytic activity in the HeLa uterine/cervix cancer cell line, mumps virus for treatment of gastric carcinoma, uterine carcinoma and cutaneous carcinoma, Newcastle Disease Virus (NDV), influenza virus for treatment of ovarian cancer, and adenovirus for treatment of for instance, cervical carcinoma (Nemunaitis J; 1999). Other reports have indicated that adenoviruses and 20 attenuated poliovirus recombinants may have use in the treatment of malignant glioma cells (Alemany R., et al 1999; Andreansky S.S., 1996), and that reovirus shows lytic capability in human U87 glioblastoma cells and NIH-3T3 cells with an activated Ras signalling pathway (Coffey M.C, et al, 1998; Strong J.E. et al, 1998).

In addition, a vaccinia oncolysate has been used in clinical trials to treat melanoma 25 (Stage II) patients (Nemunaitis J., 1999). Modified, non-neurovirulent Herpes simplex

viruses (HSV) have also been reported as showing promise for the treatment of brain tumours including intracranial melanoma, and subcutaneous human melanoma (Randazzo B.R., 1997), while adenovirus infection has been reported to enhance killing of melanoma cells by the plant mitotoxin, saporin (Satyamoorthy K., 1997).

Using viruses to target and destroy cancer cells involves complex biologies that include interactions with host cell machinery and immune response effectors (Hirasawa *et al.* 2003). Metastatic tumour spread is a pathological process associated with a series of adhesion/de-adhesion events coupled with regulated tissue degradation. It is known that adhesion to and migration through the extracellular matrix is essential for tumour invasion. The largest family of extracellular adhesion molecules is the integrin family (Marshall J.F. and Hart I.R., 1996) and members of the $\alpha\beta$ group of integrins have been shown to be expressed on a variety of cell types. For instance $\alpha_v\beta_1$ is expressed on neuroblastoma, melanoma and osteosarcoma cells, $\alpha_v\beta_3$ is expressed on melanoma, glioblastoma and renal carcinoma cells, and $\alpha_v\beta_5$ is expressed on melanoma cells as is $\alpha_v\beta_8$ (Marshall J.F. and Hart I.R., 1996).

A few of the latest additions to the growing list of oncolytic RNA viruses include strains of the common-cold virus Coxsackievirus A13, A15, A18 and A21, which are enterviruses belonging to the family *Picornaviridae* (King *et al.* 2000). The receptors involved in the binding and infection of host cells by A13, A15, A18 and A21 have been identified as intercellular adhesion molecule-1, (ICAM-1) and decay accelerating factor, (DAF) (Shafren, 1997). Binding of the Coxsackievirus to surface expressed DAF is, however, not sufficient to initiate a productive infection unless ICAM-1 is also present on the host cell surface (Shafren, 1997). ICAM-1 has been shown to be responsible for subsequent viral entry into the cell, uncoating and replication (Shafren *et al.* 1995).

Interestingly, ICAM-1 upregulation has been associated with the metastasis and disease progression in many cancers (Johnson, 1999; Natali *et al.*, 1990 and van de Stolpe & van der Saag, 1996).

Despite progress being made in the treatment of malignancies, the treatment of 5 solid tumour cancers including colo-rectal, pancreatic and ovarian cancers presents a major challenge for research and there remains the need for alternatives to existing therapy approaches.

Summary Of The Invention

10 The present invention stems from the surprising finding of significant killing of abnormal cells can be achieved with the use of a virus and the recognition/interaction of cell expressed markers utilised by the virus for infectivity of the cells.

The present inventors have surprisingly found that strains of viruses that normally cause flu-like symptoms or the common cold in animals can be used to target and kill 15 solid tumours, malignancies or cancers.

According to a first aspect, the present invention provides a method for treating a solid tumour in a subject, the method comprising administering to the subject an effective amount of a Coxsackie A group virus or a modified form thereof such that at least some cells in the tumour are killed by the virus.

20 Preferably, the solid tumour is selected from ovarian cancer, pancreatic cancer, gastrointestinal cancer, colo-rectal cancer or oesophageal cancer. Preferably, the Coxsackie A group virus is selected from the group consisting of CAV13, CAV15, CAV18, CAV21, modified forms thereof, and combinations thereof. More preferably, the Coxsackie A group virus is selected from CAV13, CAV15, CAV18, or CAV21.

In a preferred form, the Coxsackie A group virus is CAV15 or CAV21. Preferably, the Coxsackie A group virus is CAV15. More preferably the CAV15 is G-9.

In another preferred form, the Coxsackie A group virus is CAV21. More preferably, the CAV21 is Kuykendall strain.

5 In a preferred form, the Coxsackie A group virus or modified forms or combinations thereof recognise ICAM-1.

In a preferred form, the virus is a recombinant virus.

10 In another preferred form, the virus is administered to a human subject. Preferably, the virus is administered intravenously, intratumourally, intraperitoneally, intramuscularly, intraocularly, orally, transdermally or topically. More preferably, the virus is administered intratumourally or intravenously.

In a preferred form, the virus is administered in a dosage greater than about 1x10² plaque forming units per ml of innoculant. Preferably, the dosage is between 1x10² and 1x10¹⁰ plaque forming units per ml of innoculant.

15 In a preferred form, the method of the invention further comprises administering the virus to the subject in combination with an effective amount of a chemotherapeutic agent.

20 In another preferred form, the method of the invention further comprises administering the virus to the subject in combination with an effective amount of a probiotic agent.

In an embodiment of the invention the solid tumour or cancer is selected from the group consisting of ovarian cancer, pancreatic cancer, gastrointestinal cancer, stomach cancer, intestinal cancer, colorectal cancer, oral cancer, oesophageal cancer and glioma.

25 According to a second aspect, the present invention provides a pharmaceutical composition when used in treating a solid tumour or cancer in a subject, the composition comprising an effective amount of a Coxsackie A group virus or a modified form thereof together with a pharmaceutically acceptable diluent or carrier.

Preferably, the solid tumour or cancer is selected from ovarian cancer, pancreatic cancer, gastrointestinal cancer, colo-rectal cancer or oesophageal cancer.

Preferably, the Coxsackie A group virus is selected from the group consisting of CAV13, CAV15, CAV18, CAV21, modified forms thereof, and combinations thereof.

- 5 More preferably, the Coxsackie A group virus is selected from the group consisting of CAV13, CAV15, CAV18, or CAV21.

In a preferred form, the Coxsackie A group virus is CAV15 or CAV21.

Preferably, the Coxsackie A group virus is CAV15. More preferably the CAV15 is G-9.

- 10 In another preferred form, the Coxsackie A group virus is CAV21. More preferably, the CAV21 is Kuykendall strain.

In a preferred form, the Coxsackie A group virus or modified forms or combinations thereof recognise ICAM-1.

In another preferred form, the pharmaceutical composition is administered to a human subject.

- 15 Preferably, the pharmaceutical composition is administered intravenously, intratumourally, intraperitoneally, intramuscularly, intraocularly, orally, transdermally or topically. More preferably, the composition is administered intratumourally or intravenously .

20 In a preferred form, the pharmaceutical composition comprises greater than about 1×10^2 plaque forming units per ml of virus. Preferably, the composition comprises between 1×10^2 and 1×10^{10} plaque forming units per ml of virus.

In a preferred form, the pharmaceutical composition further comprises an effective amount of a chemotherapeutic agent.

25 In another preferred form, the pharmaceutical composition further comprises an effective amount of a probiotic agent.

According to a third aspect, the present invention provides use of an effective amount of a Coxsackie A group virus or a modified form thereof in a method for treating a solid tumour or cancer in a subject wherein at least some cells in the tumour are killed by the virus.

5 Preferably, the solid tumour or cancer is selected from ovarian cancer, pancreatic cancer, gastrointestinal cancer, colo-rectal cancer or oesophageal cancer.

According to a fourth aspect, the present invention provides use of an effective amount of a Coxsackie A group virus or a modified form thereof in the manufacture of a medicament for treating a solid tumour or cancer in a subject wherein at least some cells 10 in the tumour are killed by the virus.

Preferably, the solid tumour or cancer is selected from ovarian cancer, pancreatic cancer, gastrointestinal cancer, colo-rectal cancer or oesophageal cancer.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to 15 imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context 20 for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred 25 forms will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 shows immunoperoxidase staining of surface ICAM-1 expression on melanoma cells. ICAM-1 expression (white arrows) is indicated by dark cell staining;

5 Figure 2 shows relative levels of ICAM-1 and DAF expression by the melanoma cell lines Sk-Mel-28 and ME4405;

Figure 3 indicates lytic infection of two human melanoma cell lines by Coxsackievirus A21 at different time intervals post infection;

10 Figure 4 indicates lytic infection of human melanoma cells from a primary melanoma induced in a nude mouse with various doses of Coxsackievirus A21;

Figure 5 indicates lytic infection of preparations of suspension and adherent primary malignant cells from a chest wall melanoma by Coxsackievirus A21 at 20 hours post infection;

15 Figure 6 (A) indicates lytic infection of six human melanoma cell lines by Coxsackievirus A21 at twenty-three hours post infection; (B) indicates results of flow cytometric analysis of DAF (dark line) and ICAM-1 (lighter line) on the surface of human melanoma cells;

Figure 7 indicates lytic infection of different tumour cell lines by representative human enteroviruses;

20 Figure 8 indicates lytic infection of a human melanoma biopsy from lymph node by human enteroviruses Coxsackievirus A21 and B3;

Figure 9 indicates lytic infection of prostate cancer cells by selected Coxsackievirus;

25 Figure 10 shows the capacity of CAV21 and CAV15 to specifically lytically destroy melanoma cells without infecting non-melanoma cells;

Figure 11 indicates subcutaneous administration of CAV21 infected cells to NOD-SCID mice inhibits human melanoma tumour formation;

Figure 12 is a graph showing results of intratumoural treatment of preformed Sk-Mel-28 melanoma with CAV21;

5 Figure 13 is a graph showing results of intratumoural treatment of preformed Sk-Mel-28 melanoma with CAV15;

Figure 14 shows Sk-Mel-28 tumours 35 days post inoculation with PBS (left tumour) and CAV15 (right tumour); and

10 Figure 15 is a graph showing the effect of intratumoural treatment of preformed ME4405 melanoma with CAV21.

Figure 16 is a graph indicating the lytic ability of CVA21 to destroy human colon, ovarian and pancreatic tumour cells.

Mode(s) for Carrying Out the Invention

15 Although it is known that some naturally occurring Picornavirus and other viruses such as reoviruses are suitable for use in treatment of limited types of cancers, there is still a need to develop improved treatments.

20 As described herein, the present inventors have discovered that Coxsackie A group viruses can be used to lytically solid tumours or cancers. Cells that are "susceptible" are those that demonstrate induction of cytopathic effects, viral protein synthesis, and/or virus production.

The Coxsackie A group virus may be naturally occurring or modified. The Coxsackie A group virus is "naturally-occurring" when it can be isolated from a source in nature and has not been intentionally modified by humans in the laboratory. For

example, the Coxsackie A group virus can be obtained from a "field source": that is, from a human patient.

The Coxsackie A group virus may be modified but still capable of lytically infecting a solid tumour or cancer.

5 The Coxsackievirus may be a recombinant Coxsackievirus from two or more types of Coxsackieviruses with differing pathogenic phenotypes such that it contains different antigenic determinants thereby reducing or preventing an immune response by a mammal previously exposed to a Coxsackievirus subtype.

In the methods of the invention, Coxsackie A group virus is administered to a
10 solid tumour or cancer in the individual subject. A combination of different serotypes and/or different strains of Coxsackie A group virus, such as Coxsackievirus from different species of animal, can be used. If desired, the Coxsackievirus can be chemically or biochemically pretreated (e.g., by treatment with a protease, such as chymotrypsin or trypsin) prior to administration to the neoplasm. Such pretreatment
15 removes the outer coat of the virus and may thereby result in better infectivity of the virus.

The solid tumour or cancer is an abnormal tissue growth, generally forming a distinct mass, that grows by cellular proliferation more rapidly than normal tissue growth. Solid tumour cancers show partial or total lack of structural organization and
20 functional coordination with normal tissue. Preferably, at least some of the cells of the solid tumour or cancer express ICAM-1. Solid tumours or cancers that are particularly susceptible to treatment by the methods of the invention include colorectal cancer, ovarian cancer, pancreatic cancer, oesophageal cancer, stomach and intestinal cancer, etc.

The Coxsackievirus is typically administered in a physiologically acceptable carrier or vehicle, such as phosphate-buffered saline, to the solid tumour or cancer. The route by which the Coxsackievirus is administered, as well as the formulation, carrier or vehicle, will depend on the location as well as the type of the solid tumour or cancer. A wide variety of administration routes can be employed. For example, for a solid tumour cancer that is accessible, the Coxsackievirus can be administered by injection directly to the tumour. The Coxsackievirus can also be administered subcutaneously, intraperitoneally, topically, orally (e.g., for oral or esophageal cancer), or rectally (e.g., for colorectal cancer).

The Coxsackievirus is administered in an amount that is sufficient to treat the solid tumour (e.g., an "effective amount"). A solid tumour or cancer is "treated" when administration of Coxsackievirus to cells of the tumour effects oncolysis of the tumour cells, resulting in a reduction in size of the tumour, or in a complete elimination of the tumour. The reduction in size of the tumour, or elimination of the tumour, is generally caused by lysis of solid tumour or cancer cells ("oncolysis") by the Coxsackievirus. The effective amount will be determined on an individual basis and may be based, at least in part, on consideration of the type of Coxsackievirus; the individual's size, age, gender; and the size and other characteristics of the solid tumour or cancer. For example, for treatment of a human, approximately 10^2 to 10^{10} plaque forming units (PFU) of Coxsackievirus can be used, depending on the type, size and number of tumours present. The Coxsackievirus can be administered in a single dose, or multiple doses (i.e., more than one dose). The multiple doses can be administered concurrently, or consecutively (e.g., over a period of days or weeks). The Coxsackievirus can also be administered to more than one solid tumour or cancer in the same individual.

It will also be appreciated that the Coxsackievirus can be indirectly administered by using the RNA genome or a complementary DNA copy of the genome. When administered, the Coxsackievirus will still be able to replicate in the cell and cause the desired lytic infection and killing.

5 Desirably, the virus will normally be selected from Coxsackie A-group viruses. CAV21 is preferred and in particular CAV21 (Kuykendall) (Sickles G.M., Proc. Soc. Exp. Biol. Med. 102:742; Shafren D. et al J. Virol 1997, 71:4736; Hughes et al, J. Gen Virol. 1989, 70:2943; Schmidt, N.J., et al, Proc. Soc. Exp. Biol. Med., 1961, 107:63. CAV21 (Kuykendall) is available from the American Type Culture Collection (ATCC) 10 10801 University Boulevard, Manassas, Virginia 20110-2209, United States of America under Accession No. VR-850.

Typically, the patient will be treated with an initial dose of the virus and subsequently monitored for a suitable period of time before a decision is made to administer further virus to the patient pending factors such as the response of the patient 15 to the initial administration of the virus and the degree of viral infection and malignant cell death resulting from the initial treatment.

Desirably, an individual will be treated with the virus over a period of time at predetermined intervals. The intervals may be daily or range from 24 hours up to 72 hours or more as determined appropriate in each circumstance. The same or a different 20 virus may be administered each time to avoid or minimise the effect of any immune response to a previously administered virus, and a course of treatment may extend for one to two weeks or more as may be determined by the attending physician.. Most preferably, virus to which the mammal has not previously been exposed or to which the mammal generates a relatively minor immune response as may be determined by 25 standard techniques will be administered.

While readily available known viruses may be suitably employed in a method of the invention, a virus modified or engineered using conventional techniques may also be utilised. For instance, a virus may be modified to employ additional cell adhesion molecules as cell receptors. For example, Coxsackievirus A21 may be modified using 5 site-directed mutagenesis so that the peptide motif "RGD" is expressed on the viral capsid surface as is the case with Coxsackievirus A9 (CAV-9). The RGD motif is recognised by all the α_v integrin heterodimers and this capsid modification may for instance allow the virus to bind the integrin $\alpha_v\beta_3$, a cell adhesion molecule which has been shown to be up-regulated in combination with ICAM-1 on the surface of malignant 10 melanoma lesions (Natali P.G.; 1997) leading to enhanced uptake of the virus via interaction with the integrin molecule or subsequent interaction with ICAM-1. Alternatively, the virus may be modified to recognise a selectin such as E-selectin.

The invention will now be described with reference to a number of examples described below.

15

Example 1

1.1. Cell Lines

Continuous cultures of Rhabdomyosarcoma expressing ICAM-1 cells (RD-ICAM-1), HeLa-B cells, and human lung fibroblast cells (MRCS) were maintained in 20 Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal calf serum (FCS). Two melanoma cell lines Sk-Mel-28 and ME4405 were obtained from Dr. Ralph (Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia) and Dr. Peter Hersey, Cancer Research Department, David Maddison Building Level 4, Royal Newcastle Hospital, Newcastle, New South Wales, Australia, respectively. The 25 cell line Sk-Mel-28 is a metastatic melanoma cell line found to be resistant to

chemotherapeutic drugs (56). The melanoma cell culture ME4405 was established from specimens of primary melanoma lesions (69). The two melanoma cell lines were maintained in DMEM containing 10% FCS. Rhabdomyosarcoma cells (RD) a heteroploid human embryonal cell line, and HeLa-B cells an aneuploid cell clone derived from human squamous epithelial cells, were obtained from the Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia. RD cells stably transfected with cDNA encoding the immunoglobulin superfamily molecule ICAM-1 providing the RD-ICAM-1 cell line have been described elsewhere (Shafren DR, et al; 1997). MRC5 cells, derived from human lung fibroblasts were obtained from Bio-Whittaker, USA.

1.2. Viruses

Strains of CAV21 (Kuykendall strain), CAV15 (G-9) and CVB3 (Nancy) were obtained from Margery Kennett, Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia.

1.3. Virus Propagation

RD-ICAM-1 cultures (80-95% confluent) were infected with 10^4 TCID₅₀ (50% tissue culture infectious dose) of Coxsackievirus A strains according to standard procedures. Infected cells were incubated at 37°C until complete cytopathic effect was observed (within 2 days). Cells were then frozen at -80°C and thawed to release the remaining intracellular virus particles. The virus-containing medium was clarified of cellular debris by centrifugation for 5 min at 1000 x g and stored as 500 µl aliquots at -80°C. CVB3 was propagated in HeLa-B cells in the same manner as described above.

1.4 Monoclonal Antibodies (MAbs)

MAb IH4 which recognises the third SCR of DAF (24) was a gift from Dr. B. Loveland, Austin Research Institute, Melbourne, Victoria, Australia. MAb WEHI-CAM

recognises the first domain of ICAM-1 (Berendt AR, et al; 1992) and was provided by Dr. A. Boyd, Walter and Eliza Hall Institute, Melbourne, Victoria, Australia.

1.5. Flow Cytometric Analysis

Cells (1×10^6) in 100 μ l aliquots were incubated with Mab IH4 or Mab WEHI-CAM diluted in DMEM containing 1% FCS on ice for 30 min. The cells were then washed with 5.0 ml of PBS, pelleted at 1,000 $\times g$ for 5 min and resuspended in 100 μ l of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Silenus, Melbourne, Australia) diluted in PBS. Following incubation on ice for 30 min the cells were washed and pelleted, and resuspended in PBS for analysis with a FACStar analyser (Becton Dickinson, Sydney, Australia).

1.6. Colourimetric Infectivity Assay

The stock virus solutions of CAV21 and CAV15 were serially diluted 10-fold in DMEM containing 1% foetal calf serum (FCS). RD-ICAM-1 cell monolayers in 96-well plates were inoculated with 100 μ l of serial dilutions of the viruses for 48h at 37°C. To quantitate cell survival, monolayers were incubated with 100 μ l of a crystal violet-methanol solution (5% w/v crystal violet, 10% v/v methanol, 10% v/v formaldehyde solution in PBS) and washed with distilled water. The plates were read on a multiscan enzyme-linked immunosorbent assay plate reader at a wavelength of 540 nm. Fifty percent endpoint titres were calculated (Reed LJ and Muench HA; 1938) and expressed as 50% tissue culture infectious dose (TCID₅₀) per millilitre. A well was scored positive if absorbance was less than three standard deviations of the no-virus control. The TCID₅₀ for CAV21 was determined to be 2.7×10^4 units per ml while for CAV15, the TCID₅₀ was determined to be 1.6×10^4 units per ml.

1.7. Surface Expression of ICAM-1 and DAF

The relative levels of ICAM-1 and DAF expression on the surface of the melanoma cell lines SK-Mel-28 and ME4405 was determined by flow cytometric analysis. The results are shown in Fig 2.

5 As can be seen, flow cytometric analysis revealed comparable high level ICAM-1 and DAF expression on the surface of the two melanoma cell lines. A further 6 melanoma cell lines derived from metastatic melanomas also expressed high levels of ICAM-1 and DAF (data not shown). The finding of high level ICAM-1 expression on all the metastatic melanoma cells tested supports several reports in the literature noting
10 increased levels of ICAM-1 expression *in vivo* correlates with increased metastatic ability (Johnson JP, et al:1988; Kageshita T, et al:1993; Miller BE and Welch DR:1990; Natalie PG, et al:1997).

Example 2

2.1. Infection of Melanoma Cell Lines by CAV21

Monolayers of two culture-adapted melanoma cell lines Miller and MM200 were infected with CAV21 prepared in Example 1 at a multiplicity of infection of 1.0 for 1 hour prior to removal of the inoculum and the cells incubated in culture medium (DMEM containing 1% foetal calf serum and penicillin streptomycin) for 24 hours at
20 37°C.. The results shown in Figure 3 indicate that CAV21 was able to induce significant changes in the cellular cytopathology of both cell lines as early as five hours post infection (PI) and by nine hours PI almost complete killing of all the melanoma cells.

Example 3

3.1. Infection of Melanoma Cells from Primary Melanoma by CAV21

Cells from a primary melanoma removed from a nude mouse that had been previously subcutaneously inoculated with human melanoma cells from cell line ME 5 4405 using conventional methods, were highly susceptible to CAV21 infection and killing, even at a challenge rate of 0.005 CAV21 particles per melanoma cell as shown in Figure 4.

Example 4

4.1. Infection of Melanoma Cells Isolated From Tissue Biopsy by CAV21

Melanoma cells were isolated from fresh biopsy of a primary chest wall melanoma by the "spilling" technique and by digestion in collagen-trypsin and DNAase. Briefly, cells were released from the melanoma biopsy by macerating the biopsy with the plunger of a 10ml syringe. The resulting melanoma cell suspension was purified on a Ficol-Hypaque (Amersham Pharmacia, Uppsala, Sweden) gradient. Contaminating fibroblasts and leucocytes were removed by mixing with Dynal beads coated with monoclonal antibodies (Mab's) to human fibroblasts (Cat#; MAS516X, SeraLab) and to the leucocyte common antigen (CD45, Cat# 17-0804-3, Amrad Biotech, Victoria, Australia).

Subsequently, 1×10^6 cells were placed into wells of a 24-well tissue culture plate and inoculated with approximately 1×10^5 plaque forming units of CAV21 prepared in Example 1. Following incubation at 37°C for 20 hours, cells were assessed for cell death by staining with propidium iodine and microscopic analysis.

Figure 5 shows that both adherent and suspension primary melanoma cells were efficiently killed as a result of CAV21 infection during the 20 hour incubation period.

Example 5

5.1 Expression of ICAM-1 and DAF on Melanoma Cells Susceptible to CAV21 Infection

5 To confirm melanoma cells are highly susceptible to infection and resultant killing by CAV21, six additional human melanoma cell lines derived from primary human melanomas were infected with CAV21 prepared in Example 1.

Figure 6(A) indicates that all melanoma cell lines except one (ME 105) were killed as a result of CAV21 infection during a 23 hour incubation period.

10 To confirm high level expression of ICAM-1 and DAF on the surface of malignant melanoma cells, cells from each cell line were treated with the Mab IH4 and Mab WEHI-CAM. The binding of the anti-DAF and anti-ICAM-1 Mab was detected by flow cytometric analysis as described above. The fluorescence histograms shown in Figure 6(B) confirm high level expression of DAF and ICAM-1 on the surface of all melanoma cell lines examined except the ME 105 cell line. The lack of DAF and ICAM-1 expression rendered this cell line refractile to CAV21 infection.

Example 6

6.1 Selective Infection of Melanoma Cells Expressing ICAM-1

20 To highlight the selective nature of CAV21 infection of ICAM-1 expressing human melanoma cells, monolayers of melanoma cell line MM 200 were inoculated with approximately 1×10^5 plaque forming units of CAV21, Coxsackievirus B3 (CVB3), Echo virus type 7 (E7) or Coxsackievirus B1 (CVB1) in wells of a 24-well tissue culture plate for one hour at 37°C, respectively. The viral inoculate was subsequently removed and the cell monolayers then washed with phosphate buffered

saline (PBS), and 1.0 ml of DMEM containing 1.0% foetal calf serum was added to each well and the cells incubated at 37°C for 48 hours. To quantitate cell survival, monolayers were incubated with a crystal violet/methanol solution, washed with distilled water and microscopically examined at 100 X.

5 Figure 7 shows that following the 48 hour incubation period only CAV21 infected the MM 200 melanoma cells while the reverse occurred in the rhabdomyosarcoma cells (RD) where CVB1, CVB3 and E7 infection and killing is evident. RD cells express DAF but no ICAM-1. However, when ICAM-1 is expressed on the surface of RD cells they are highly susceptible to CAV21 induced infection and killing.

10

Example 7

7.1 Infection of Melanoma Biopsy With CAV21

Sections of solid human melanoma lymph node biopsies were placed in wells of a 24-well tissue culture plate and mock infected or challenged with approximately 1×10^5 plaque forming units of CAV21 or CVB3.

15 The results shown in Figure 8 indicate that CAV21 infection resulted in severe tissue destruction around the perimeter of the melanoma biopsy treated with that virus while no detectable viral membrane destruction was observed in the mock and CVB3 infected biopsies.

20

Example 8

8.1. Lytic Infection of Human Melanoma Cells by CAV21 and CAV15

To assay the oncolytic potential of CAV15 and CAV21 on human melanoma cell lines, Sk-Mel-28 and ME4405 cells were seeded into flat-bottom 96-well microtiter plates (Becton Dickinson) at 3×10^4 cells per well. Following incubation for 24 h at

37°C, culture medium was removed and replaced with fresh medium containing the appropriate viral serial dilution in a final volume of 100 µl. Stock viral preparations were serially diluted 10⁻¹ through to 10⁻⁷. Following viral inoculation, the plates were incubated at 37°C for 48 h and cell survival was detected by crystal violet staining as 5 described above.

All three cell lines RD-ICAM-1, Sk-Mel-28 and ME4405 were found to be permissive to lytic infection by both CAV21 and CAV15. Following an incubation period of 48 h, the no virus control showed no signs of viral induced CPE while extensive cell lysis was observed across all cell cultures at a dilution of 10⁻¹ and 10⁻². At 10 higher viral dilutions Sk-Mel-28 cells were shown to be more permissive to viral lysis compared to ME4405 and RD-ICAM-1 cell lines.

The overall oncolytic potential of CAV21 and CAV15 was higher in the melanoma cell lines, compared to the control RD-ICAM-1 cells. While all cell types express similar levels of ICAM-1, DAF expression in RD-ICAM-1 cells is significantly lower than on melanoma cells (see Figure 2) accounting for lower viral attachment via 15 DAF to RD-ICAM-1 cells. DAF has previously been shown to be a low affinity sequestration molecule for many Coxsackieviruses, assisting the capture of virus particles and hence infectivity of the cells(Lea SM, et al; 1998). The presence of higher levels of DAF expression on the melanoma cell lines compared to the RD-ICAM-1 cells 20 increases the probability of viral access to ICAM-1 receptors, thus leading to an increased level of infection and cell lysis.

8.2. Lytic Infection of Human Prostate Cancer Cells by Coxsackievirus

Cells from the human prostate cancer cell line CP3 (which expresses ICAM-1) were seeded into a flat-bottom 96-well microtitre plate (Becton Dickenson) at 3 x 10⁴ 25 cells per well and treated with serial dilutions of CAV13, CAV15, CAV21 and the

Coxsackievirus B-group virus CVB3 following incubation of the cells, as described in Example 8.1 above. PC3 cells are available from the American Type Culture Collection (ATCC) Manassas, Virginia, USA under Accession No. CRL-1435.

As shown in Figure 9, the PC3 cells were highly permissive to lytic infection by 5 CAV15. Extensive lytic infection was also observed for both CAV13 and CAV21.

8.3. Selective Replication of CAV21 and CAV15 in the Human Melanoma Cell Lines

Sk-Mel-28 and ME4405

The selectivity of CAV21 and CAV15 for the melanoma cell lines Sk-Mel-28 and ME4405 was studied using an *in vitro* specificity assay.

10 Sterile cell culture inserts were used to divide the wells of a standard six well plate tissue culture plate. Inside the cell culture insert, either Sk-Mel-28 cells or ME4405 cells were grown, with MRC5 or RD cells grown around the cell culture insert. Once the cells had adhered, the cell culture inserts were removed from each of the well allowing the cell culture media to evenly cover the co-culture. When the perimeters of 15 both cell populations had fused, the co-cultures were washed twice with PBS and then inoculated with 500 µl of either PBS or stock virus (10^5 TCID₅₀) for 1 h at 37°C. Following incubation at 37°C, fresh DMEM containing 1% FCS was added to each of the wells and the plates incubated for 48 h at 37°C in a 5% CO₂ atmosphere. Cell monolayers were monitored by light microscopy for signs of virus-induced CPE, prior to 20 each well being stained with 3 ml of crystal violet solution for the detection of cell survival from viral induced lytic infection. The capacity of CAV21 and CAV15 viruses to specifically lytically destroy melanoma cells without infecting non-melanoma surrounding cells is illustrated in Figure 10.

As can be seen, the inner cultures of melanoma cells in each well treated with 25 CAV21 or CAV15 were totally destroyed by the viruses, but were unaffected by CVB3

virus which does not employ ICAM-1 as a receptor for cell entry. CVB3 which employs the Coxsackie-and adenovirus receptor (CAR) for cell entry (10). MRC5 cells appeared to be refractory to lytic infection by both CAV21 and CAV15. These cells are derived from a human lung fibroblast culture and only express low levels of ICAM-1 (unpublished data). The present data shows that rapid and effective lytic infection of target cells facilitated high level ICAM-1 and DAF expression. RD cells, which do not express ICAM-1, were not destroyed by either CAV21 or CAV15 infection. Furthermore, the results show little if any spread of CAV21 and CAV15 to receptor negative cells that are in direct contact with virally infected receptor-bearing cells.

10

Example 9

The lytic infection of preformed melanoma tumours *in vivo* was evaluated by a series of animal challenge experiments using NOD-SCID mice.

9.1. Development of Melanoma Xenografts in NOD-SCID Mice

15 All animal work was performed under guidelines approved by The University Of Newcastle Animal Care and Ethics Committee. NOD-SCID mice were housed in pathogen-free quarters in the animal handling facility located at the David Maddison Building, Level 5, Newcastle, NSW, Australia.

Sk-Mel-28 and ME4405 cells were grown in DMEM containing 10% FCS. The 20 cells were harvested and washed twice with DMEM, and resuspended in sterile PBS. The cell concentration of the suspension was determined with a haemocytometer and cell viability was assessed by trypan blue staining. Only cell preparations with >95% viability were used for xenotransplantation. Prior to xenotransplantation, animals were anaesthetised with intraperitoneal (i.p) injections of Rompun/Ketamine (50 mg/kg). For

the monitoring of animals and measurement of tumour growth, animals were anaesthetised with 3% isofluorane.

The tumour cells were xenografted into the flank of anaesthetised 4-6 week old female NOD-SCID mice. Xenograft tumour growth was observed daily and measured 5 with callipers at various intervals with all measurements recorded in millimetres over the course of 5 weeks. Estimates of tumour volumes were calculated using known methods (Davies CD, et al; 1997).

9.2. Subcutaneous Viral Delivery

In a preliminary experiment employing fifteen NOD-SCID mice, the local 10 subcutaneous delivery of virus through *ex vivo* infected cells was assessed for inhibition of tumour growth. The mice in the control group (n=5) were injected subcutaneously with Sk-Mel-28 cells (1×10^7) cells at individual sites in both the upper and lower flank. The CAV21 group (n=5) received an injection of 1×10^7 Sk-Mel-28 cells in the upper flank and a second injection of Sk-Mel-28 (1×10^7) cells that had been pre-incubated with 15 10^4 TCID₅₀ of CAV21 at room temperature for 1 hour *ex vivo*. The CAV15 group (n=5), was treated the same as the CAV21 group except that the second injection in the lower flank contained Sk-Mel-28 (1×10^7) cells that had been incubated with 10^4 TCID₅₀ of 20 CAV15. Four weeks post-injection, a representative of the control group was sacrificed and shown to bear two individual tumour masses corresponding to the two injections sites of the Sk-Mel-28 (1×10^7) cells. In contrast a representative of the CAV21 group beared no detectable tumour formation in either the uninfected cell or virally infected cell sites of injection (Figure 11). Upon autopsy examination, all remaining members of the control group were shown to possess two distinct melanoma xenograft tumour growths, while remaining members of the CAV21 group (17 weeks post injection)

exhibited no detectable tumour growth in either site of injection. Mice in the CAV15 group exhibited no tumour formation at 4 weeks post- injection.

9.3. Intratumoural Viral Delivery

Twenty NOD-SCID mice were injected with Sk-Mel 28 cells (1×10^7) in the upper flank. When the tumour volume reached $\sim 50-100 \text{ mm}^3$ the animals were randomly divided into groups of five and housed in separate cages. Groups of mice were injected intratumourally with 100 μl of active CAV21 or CAV15 containing $10^{3.2}$ or $10^{4.2}$ TCID₅₀ doses, respectively. The remaining animals received 100 μl of PBS injected directly into the xenografts. The different treatment groups were housed in individually vented cages maintained under negative pressure, ensuring that virus and other pathogens were contained within the individual cages.

A dose of $10^{3.2}$ or $10^{4.2}$ TCID₅₀ of either CAV21 or CAV15 respectively, was sufficient to produce significant tumour reduction in animals bearing preformed Sk-Mel-28 tumours at 14 days post-injection. The trend of reduction of tumour burden continued for the next 14-21 days. No detectable tumours were observed at 30-35 days post-injection (see Figures 12 and 13). The difference observed between the CAV21 treated group and the PBS treated control group was statistically significant ($P=0.0023$, t test). Animals bearing Sk-Mel-28 tumours and injected with CAV21 showed no clinical signs of CAV21 illness. The capacity of CAV15 to drastically reduce melanoma tumour burden is shown in Figure 14. At 35 days post-injection, the melanoma xenograft treated with PBS was approximately 2037 mm^3 while the CAV15 treated tumour was approximately 2 mm^3 in volume ($P=0.0053$, t test). The CAV15 treated tumour shown comprises mostly residual connective tissue.

9.4. Intratumoural Delivery of CAV21 to ME4405 Xenograft

The intratumoural delivery of CAV21 to a different melanoma (ME4405) xenograft was undertaken to further confirm the anti-tumour therapy potential of this virus. Fifteen NOD-SCID mice were injected with ME4405 cells (5×10^6) 5 subcutaneously in a single site on the flank. When tumour volumes had reached approximately 500 mm^3 , the animals were randomly divided into groups of five and housed in separate cages. Five animals were injected intratumourally with $100 \mu\text{l}$ of active CAV21 containing $10^{3.2} \text{ TCID}_{50}$ doses, while five mice received $100 \mu\text{l}$ of PBS injected directly into the xenografts and the remaining five mice were left untreated. As 10 shown in Figure 15, intratumoural administration of CAV21 was able to markedly reduce tumour development of ME4405 cells within 25 days post-injection even though the initial pre-injection tumour volume was 5-fold greater than those utilised above. The ME4405 xenografts were observed to be more aggressive than the Sk-Mel-28 tumours as assessed by significantly faster growth rates of tumours in the control groups.

15 The ME4405 cell line generated highly vascular aggressive tumours compared to Sk-Mel-28 tumours which grew at a slower rate and were not as vascular as the ME4405 tumours.

In contrast to mice bearing Sk-Mel-28 xenografts, when CAV21 was injected 20 into animals with ME4405 tumours, some signs of illness were observed, the most notable being a transient weakness in both the fore and hind limbs. No positional abnormalities were observed.

Example 10

10.1. CVA21 oncolytic infectivity assay

Monolayers of ovarian cancer cell lines (JAM and OVHS-1), pancreatic cancer 25 cells lines (Panc-1 and AsPC-1) and the colon cancer cell line (LIM2537) propagated in

96-well tissue culture plates were inoculated with 10-fold serial dilutions (100 µl/well in quadruplicate) of Coxsackievirus A21 (CVA21) in DMEM containing 1% fetal calf serum (FCS) and incubated at 37°C in a 5% CO₂ environment for 72 h. The oncolytic capacity of CVA21 in these cancer cell lines was expressed as an 50% percent infectious 5 end point titre (Figure 16). The end-point titres were calculated using the method of Reed and Muench by scoring cell monolayers that exhibited detectable (microscopic examination) cytopathic effect (CPE) as positive for viral infection.

Summary

10 This study demonstrates that CAV13, CAV15 and CAV21 have the capacity to lytically destroy malignant cell lines.

Specifically, the *in vitro* analysis of CAV21 and CAV15 infection of melanoma cells shows that these two viruses are able to selectively infect Sk-Mel-28 and ME4405 cell lines as a result of the expression of ICAM-1 and DAF while each of the 15 Coxsackieviruses mentioned above were able to infect and cause the death of cells of the prostate cancer line PC3. Moreover, the intratumoural injection of CAV21 and CAV15 into xenografts of human melanoma cell lines grown in the flanks of NOD-SCID mice show that CAV21 and CAV15 possess therapeutic applications against malignant melanoma. The direct injection of either of the two viruses into pre-formed melanoma 20 tumours suppressed tumour growth and led to significant tumour regression and in some cases complete tumour destruction compared to control animals. Furthermore, the delivery of cells infected by virus *ex vivo* yielded total inhibition of tumour growth and demonstrates that *ex vivo* CAV21 infected melanoma cells are capable of delivering sufficient virus to inhibit local tumour growth. In addition, injection of infected cells

subcutaneously in a distant region to the initial tumour challenge shows that the virus can travel systemically.

5 *In vitro* cell cultures of ovarian, pancreatic and colon cancers were propagated as cell monolayers and incubated with increasing doses of CAV21 ranging from 10^7 - 10 TCID₅₀. Following incubation for 72 hours at 37°C, microscopic examination revealed that challenge with CAV21 induced significant levels of lytic cell oncolysis in all cultures of ovarian, pancreatic and colon cancers (Figure 16).

10 The pathogenesis of CAV21 and CAV15 infections are mainly asymptomatic or manifest by no more than minor malaise. The Coe strain of CAV21 has recently been approved for live administration by the Food and Drug Administration (FDA) of the United States of America for the clinical assessment of specific anti-viral agents against CAV21 (90). The recent development of specific antiviral agents against CAV21 and CAV15 provides the added safety precaution of drug intervention to control viral infection.

15 In addition to the targeting and oncolysis of malignant cells by CAV13,15,18 and 21 via interactions with surface expressed ICAM-1, DAF or both ICAM-1 and DAF , the susceptibility such lines to destruction by the above viruses many also be due to a cellular defect in their interferon activation pathway which prevents such cells inhibiting viral infection by the action of cellular interferon (34).

20 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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The claims defining the invention are as follows:

1. A method for treating solid tumour or cancer in a subject, the method comprising administering to the subject an effective amount of a Coxsackie A group virus or a modified form thereof such that at least some cells in the tumour or cancer are killed by the virus.
2. The method according to claim 1 wherein the solid tumour or cancer is selected from the group consisting of ovarian cancer, pancreatic cancer, gastrointestinal cancer, stomach cancer, intestinal cancer, colo-rectal cancer, oral cancer, oesophageal cancer and glioma.
3. The method according to claim 1 or 2 wherein the Coxsackie A Group virus is selected from the group consisting of CAV 13, CAV15, CAV18, CAV21, modified forms thereof, and combinations thereof.
4. The method according to any one of claims 1 to 3 wherein the Coxsackie A group virus or modified forms or combinations thereof recognise ICAM-1.
5. The method according to any one of claims 1 to 4 wherein the Coxsackie A group virus is administered intravenously, intratumourally, intraperitoneally, intramuscularly, intraocularly, orally, transdermally or topically.
6. The method according to any one of claims 1 to 5, wherein said method further comprises administering said virus to said subject in combination with an effective amount of a chemotherapeutic agent.
7. The method according to any one of claims 1 to 6, wherein said method further comprises administering said virus to said subject in combination with an effective amount of a probiotic agent.
8. A pharmaceutical composition when used in treating a solid tumour or cancer in a subject, the composition comprising an effective amount of a Coxsackie A group virus or a modified form thereof together with a pharmaceutically acceptable diluent or carrier.
9. The pharmaceutical composition according to claim 8, wherein the solid tumour or cancer is selected from the group consisting of ovarian cancer, pancreatic cancer, gastrointestinal cancer, stomach cancer, intestinal cancer, colo-rectal cancer, oral cancer, oesophageal cancer and glioma.
10. The pharmaceutical composition according to claim 8 or 9 wherein the Coxsackie A group virus is selected from the group consisting of CAV13, CAV15, CAV18, CAV21, modified forms thereof, and combinations thereof.

11. The pharmaceutical composition according to any one of claims 8 to 10, wherein the Coxsackie A group virus or modified forms or combinations thereof recognise ICAM-1.
12. The pharmaceutical composition according to any one of claims 8 to 11, formulated for administering intravenously, intratumourally, intraperitoneally, intramuscularly, intraocularly, orally, transdermally or topically.
13. The pharmaceutical composition according to any one of claims 8 to 12, further comprising an effective amount of a chemotherapeutic agent.
14. The pharmaceutical composition according to any one of claims 8 to 13, further comprising an effective amount of probiotic agent.
15. Use of an effective amount of a Coxsackie A group virus or a modified form thereof in a method for treating a solid tumour or cancer in a subject wherein at least some cells in the tumour are killed by the virus.
16. The use according to claim 15, wherein said solid tumour or cancer is selected from the group consisting of ovarian cancer, pancreatic cancer, gastrointestinal cancer, stomach cancer, intestinal cancer, colo-rectal cancer, oral cancer, oesophageal cancer and glioma.
17. Use of an effective amount of a Coxsackie A group virus or a modified form thereof in the manufacture of a medicament for treating a solid tumour or cancer in a subject wherein at least some cells in the tumour are killed by the virus after administration.
18. The use according to claim 17, wherein said solid tumour selected from the group consisting of ovarian cancer, pancreatic cancer, gastrointestinal cancer, stomach cancer, intestinal cancer, colo-rectal cancer, oral cancer, oesophageal cancer and glioma.
19. The use according to any one of claims 15 to 18, wherein the Coxsackie A group virus is selected from the group consisting of CAV13, CAV15, CAV18, CAV21, modified forms thereof, and combinations thereof.
20. The use according to any one of claims 15 to 19 wherein the Coxsackie A group virus or modified forms or combinations thereof recognise ICAM-1.
21. The use according to any one of claims 15 to 20 wherein the Coxsackie A group virus is administered intravenously, intratumourally, intraperitoneally, intramuscularly, intraocularly, orally, transdermally or topically.
22. The use according to any one of claims 15 to 21, wherein said medicament is suitable for use in combination with an effective amount of chemotherapeutic agent.

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23. The use according to any one of claims 15 to 22, wherein said medicament is suitable for use in combination with an effective amount of probiotic agent.

24. A method of treating a solid tumour or cancer in a subject, the method comprising administering to the subject a pharmaceutical composition according to any one of claims 8 to 14 such that at least some cells in the tumour or cancer are killed by the virus.

25. The method according to claim 24 wherein said solid tumour or cancer is selected from the group consisting of ovarian cancer, pancreatic cancer, gastrointestinal cancer, stomach cancer, intestinal cancer, colo-rectal cancer, oral cancer, oesophageal cancer and glioma.

Dated 30 May, 2007

Virotarg Pty Ltd

Patent Attorneys for the Applicant/Nominated Person

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Figure 1

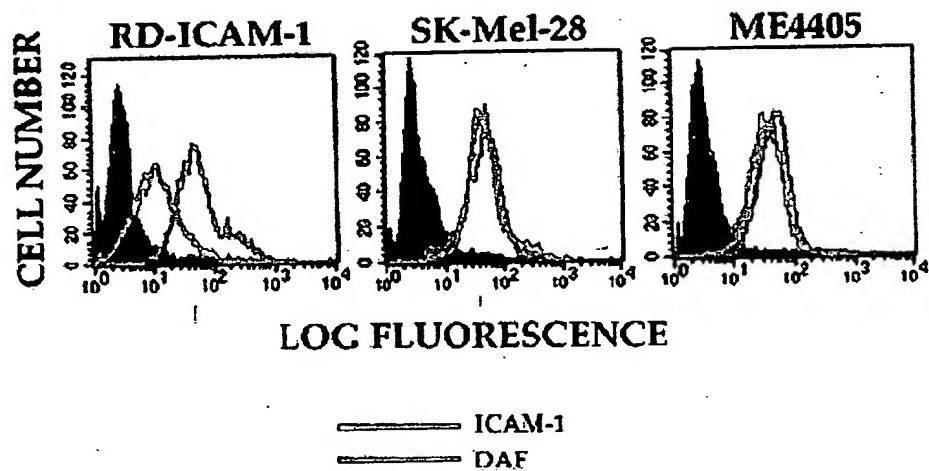


Figure 2

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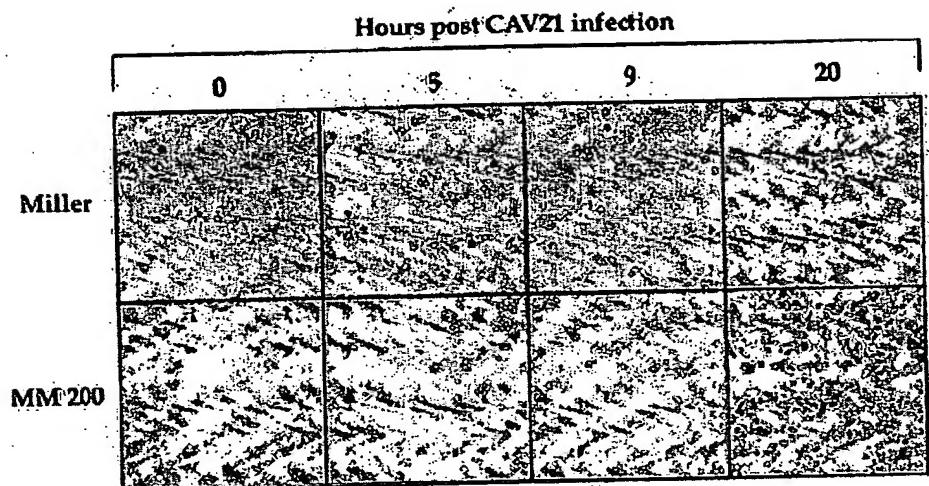


Figure 3

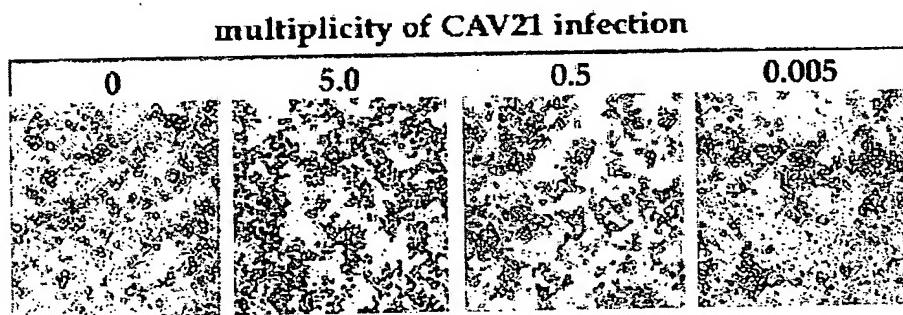


Figure 4

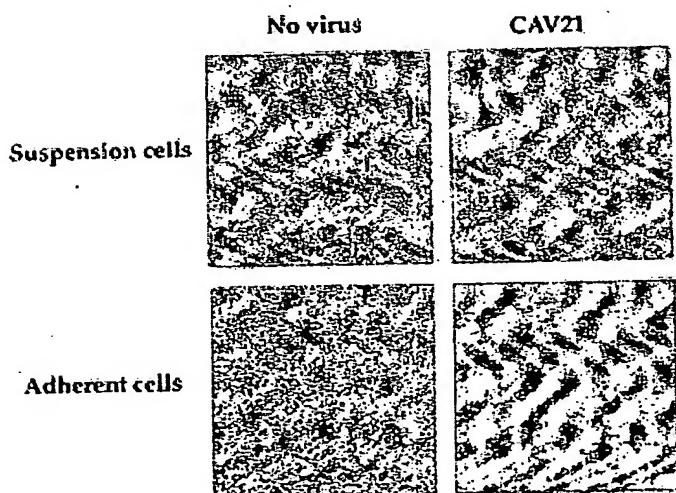


Figure 5

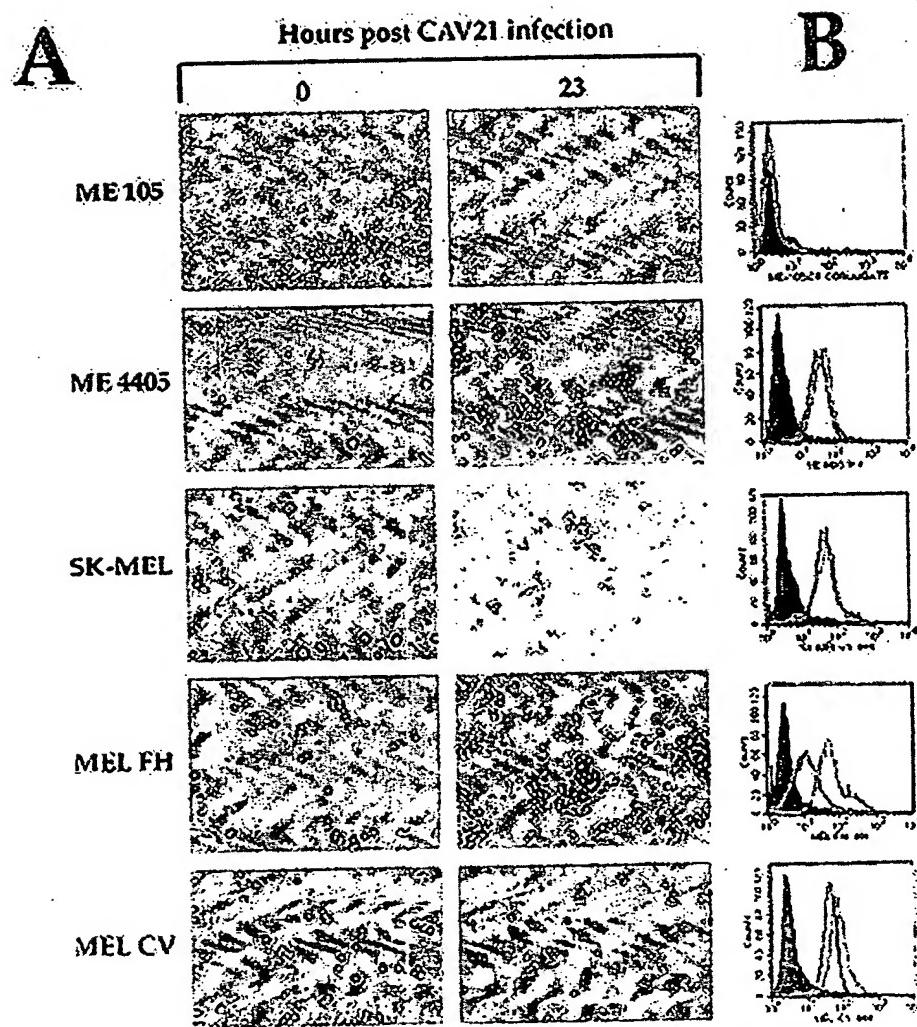


Figure 6

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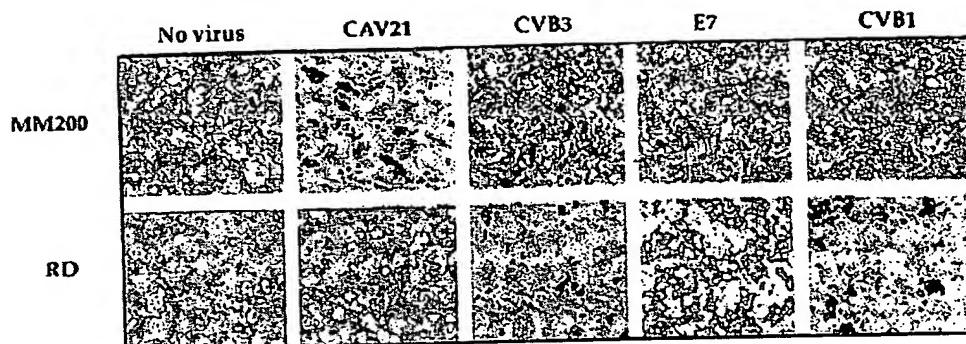


Figure 7

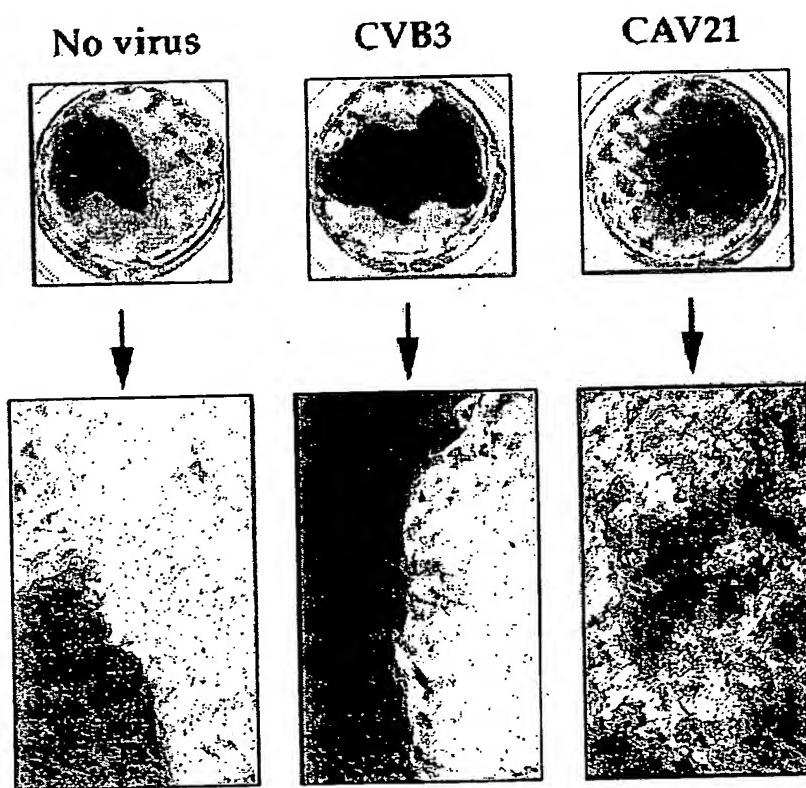


Figure 8

Oncolytic Potential of Viruses on PC3 cells

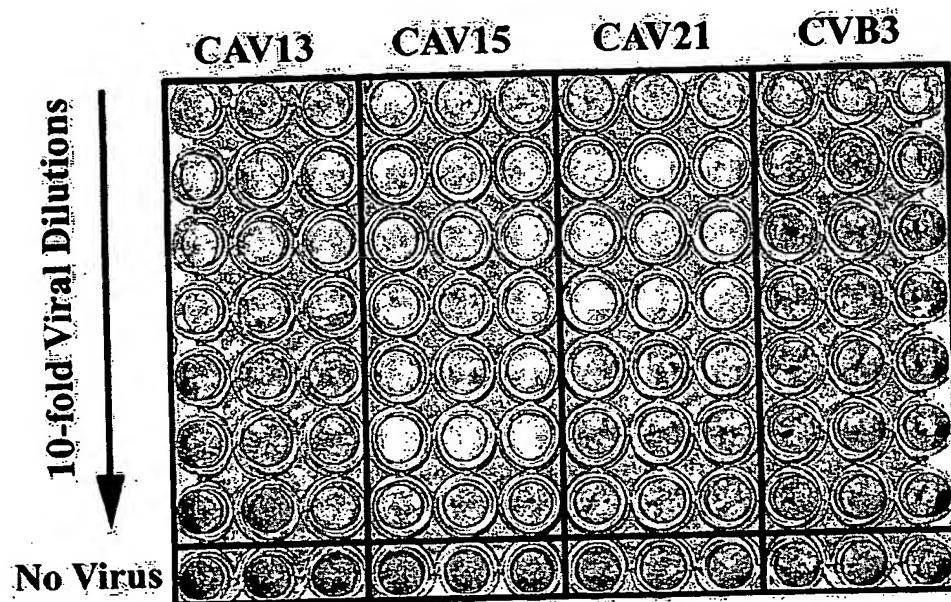


Figure 9

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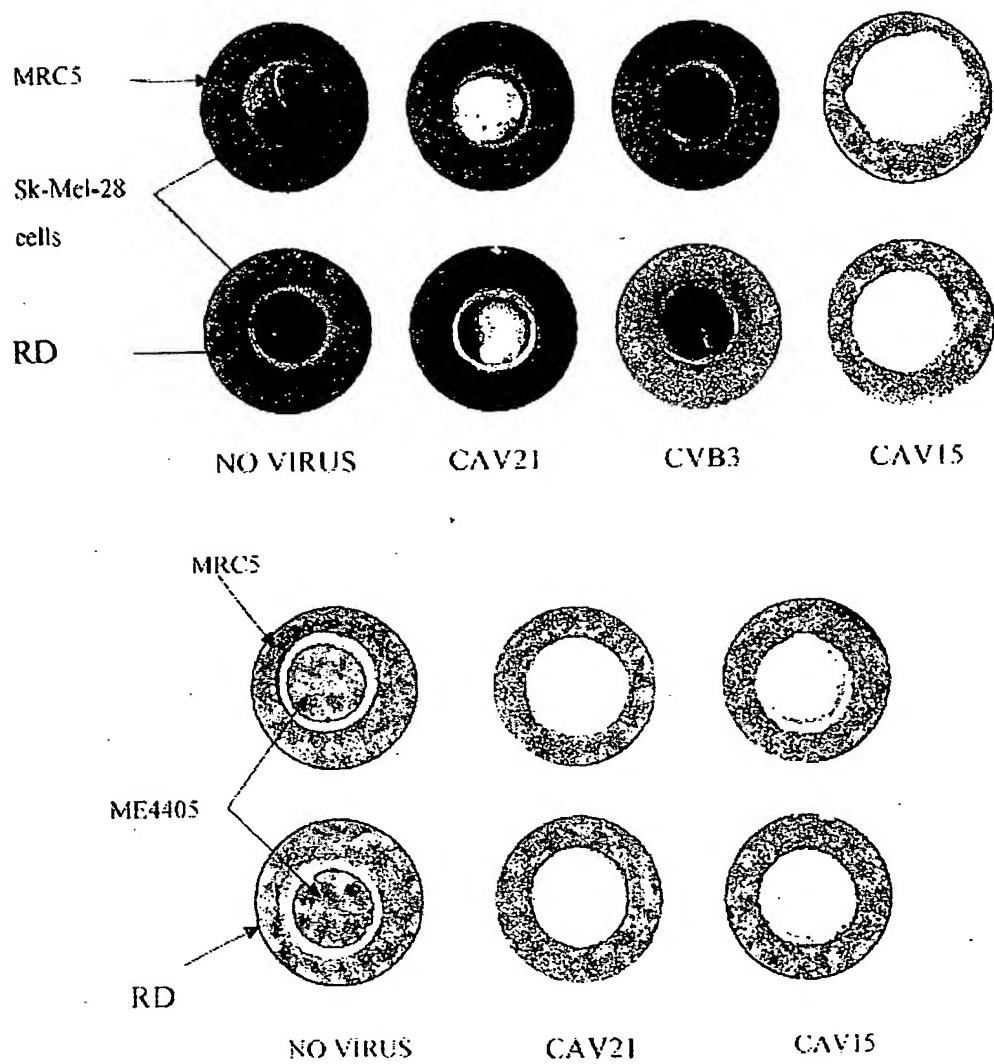


Figure 10

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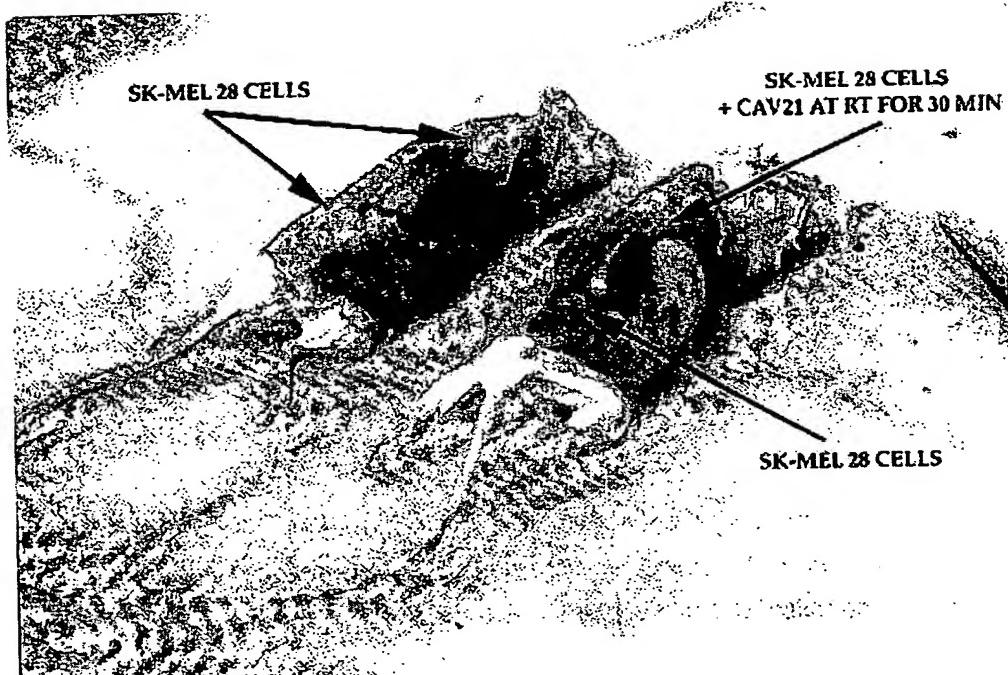


Figure 11

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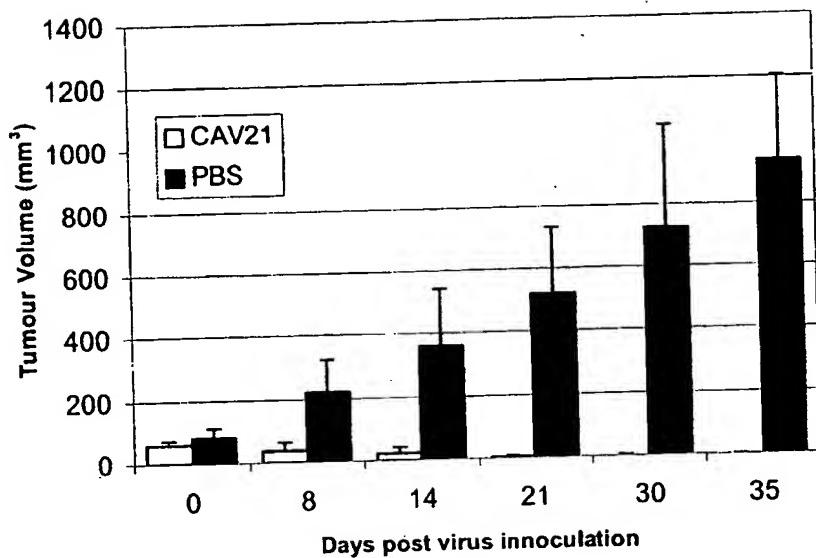


Figure 12

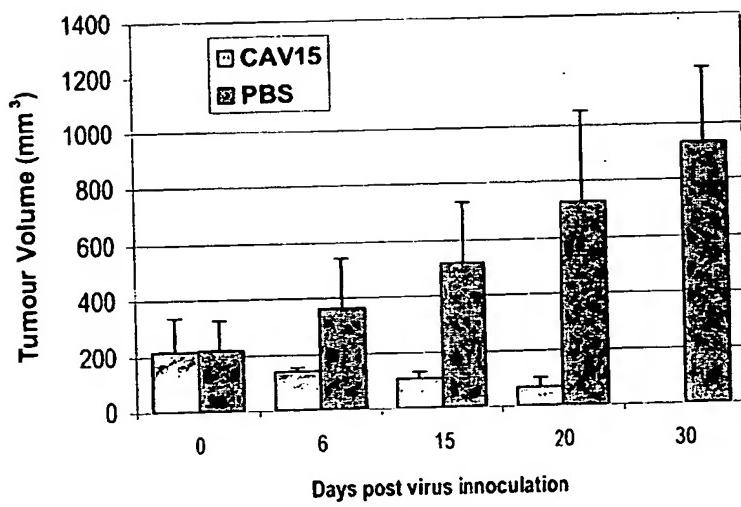


Figure 13

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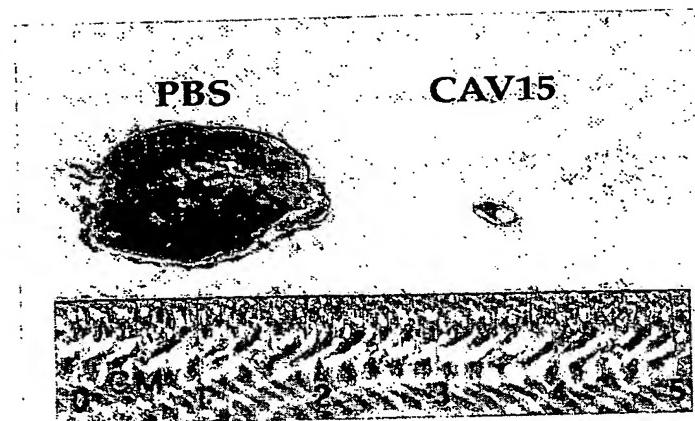


Figure 14

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Intratumoural treatment of preformed Me4405 tumours with CAV21

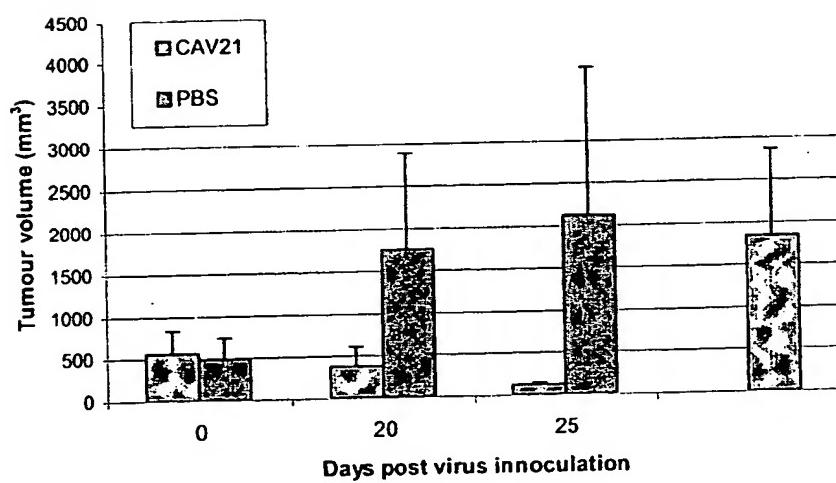
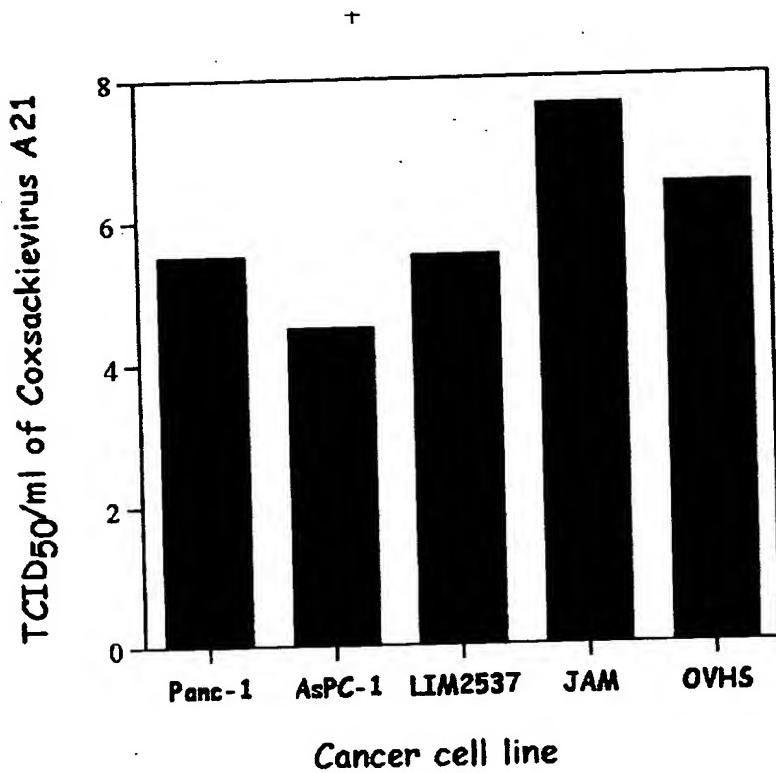


Figure 15

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Figure 16